

Aus dem Institut für Virologie der Universität Zürich

(Direktor: Prof. Dr. R. Wyler)

Arbeit unter Leitung von Dr. E. Peterhans

THE CHEMILUMINESCENT RESPONSE
OF BOVINE POLYMORPHONUCLEAR LEUCOCYTES
ISOLATED FROM MILK AND BLOOD

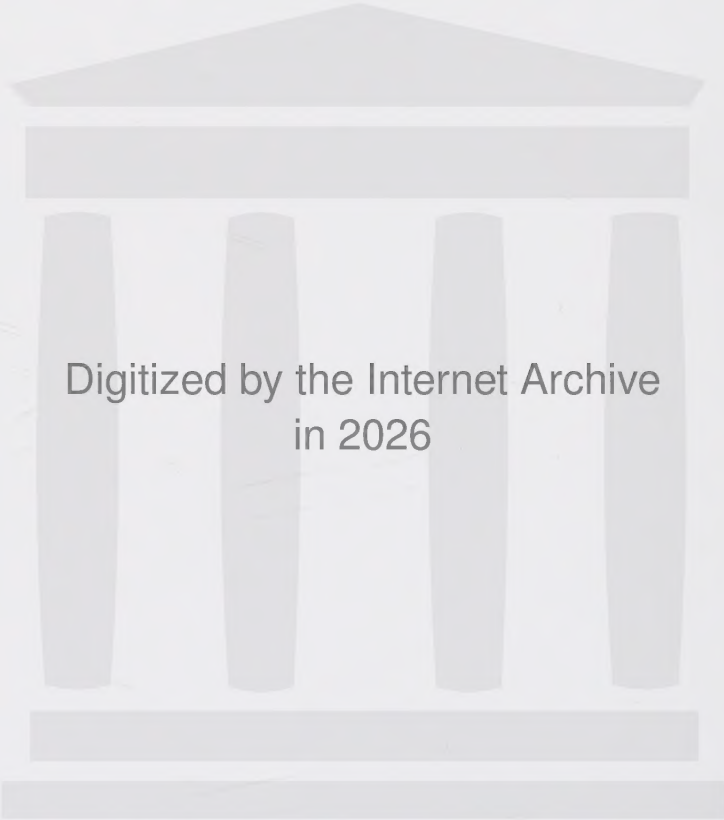
Inaugural-Dissertation
zur Erlangung der Doktorwürde
der Veterinär-Medizinischen Fakultät
der Universität Zürich

vorgelegt von
Leonhard Weber
Tierarzt
von Langwies (GR)

Genehmigt auf Antrag von
Prof. Dr. R. Wyler, Referent
Prof. Dr. E. Jenny, Korreferent

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SUMMARY

Polymorphonuclear leucocytes (PMN) were isolated from milk and blood of healthy cows, and the generation of reactive oxygen by the two cell populations was compared by measuring chemiluminescence (CL) after stimulation with zymosan. The ratio of milk to blood PMN CL was relatively constant in a given animal, but varied widely between different cows, ranging from 0.3 to 1.3.

The relative contributions of various oxygen species to CL was studied by measuring quenching using specific oxygen scavengers. While the relative contributions of H_2O_2 , O_2^- and $^1\text{O}_2$ seemed to be similar in both milk and blood PMN, the $\text{OH}\cdot$ radical was clearly more prominent in PMN isolated from milk than from blood. In addition, blood PMN CL was more dependent on the presence of glucose in the reaction medium than milk PMN CL. Furthermore, the CL response to phorbol myristate acetate, to the Ca^{2+} ionophore A 23187 and to Sendai virus was different in the two cell types. The results suggest that CL generation in milk PMN differs from that in blood PMN in quantitative as well as qualitative aspects.

INTRODUCTION

A variety of tests have been used to evaluate the function of polymorphonuclear leucocytes (PMN), e.g: the measurement of the rate at which bacteria are phagocytosed and killed, as well as more direct estimates of PMN metabolism such as the O_2 -uptake and nitroblue tetrazolium (NBT) dye reduction assays and the measurement of hexose monophosphate (HMP) shunt activity (7,17,19,21). More recently, the measurement of chemiluminescence (CL) has been shown to be a simple and sensitive assay of PMN activity (12,18).

On activation by particulate or soluble stimulants, PMN produce a variety of highly unstable oxygen metabolites such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($'O_2$) and the hydroxyl radical ($OH\cdot$) (25,26).

These oxygen species are essential factors in the efficient killing of bacteria by PMN (3,10,11,28). On reaction with easily oxidized substrates, light is emitted as a result of electronic relaxation.

However, while giving an overall estimate of the cell's capacity to generate "activated" oxygen, the measurement of CL does not allow conclusions as to the biochemical reactions underlying cellular light emission. In addition to the glucose-dependent reactions of the classical respiratory burst, pathways of the arachidonic acid cascade have recently been shown to lead to CL (23). This suggests that, in addition to reactions directly related to the

killing of bacteria (4,11,27), CL also reflects the activity of pathways related to the production of prostaglandins, thromboxanes and leucotrienes. This suggests that several metabolic activities of PMN can result in cellular light emission, only some of which might correlate with other assays of PMN activity. In addition, the nature of the chemiluminescent response is known to depend on the agent used to activate the cells (23). Therefore, any conclusions as to the functional state of PMN must remain tentative unless the source of cellular light emission can be attributed to defined biochemical reactions. As a step into this direction, we have investigated several aspects of CL generation by bovine blood PMN and PMN isolated from milk, which are known to be less efficient phagocytes than are blood PMN (8,14,15,17, 21). The results demonstrate that i) the ratios between milk and blood PMN CL are typical for individual cows and ii) the biochemical reactions contributing to CL may vary in blood and milk PMN as suggested by different contributions of glucose-dependent and -independent reactions to cellular light emission as well as by differences in the spectrum of oxygen species contributing to CL. Moreover, the chemiluminescent response to zymosan, phorbol myristate acetate and the Ca^{2+} ionophore A 23187 is generally more pronounced in blood PMN than in milk PMN while the reverse is true in response to a Paramyxovirus, Sendai Virus.

MATERIALS AND METHODS

Chemicals:

Luminol, catalase (12'000 units/mg), superoxide dismutase (3'000 units/mg), Oyster glycogen, Type II, phorbol myristate acetate (PMA) and zymosan A were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Sodium benzoate was purchased from Fluka AG, Buchs, Switzerland, and sodium azide, D-glucose and lactose were supplied by E. Merck AG, Darmstadt, Federal Republic of Germany. The Ca ionophore A 23187 was a gift from Dr. R. L. Hamill, Eli Lilly Co., Indianapolis, Indiana, USA.

Preparation of PMN

Solutions used in the isolation of PMN were sterilized by filtration (Millipore 0.22 μ m filter). All milk and blood samples were taken from healthy Swiss Braunvieh and Simmenthal cows at different stages of lactation. Blood PMN were isolated according to the procedure of Carlson and Kaneko (5): forty ml of venous blood was withdrawn into 5 ml of sterile phosphate buffered saline (PBS, pH 7.2), containing 1.5% Ethylenediaminetetraacetic acid (EDTA) in a 50 ml Corning centrifuge tube (Art.Nr. 4.988.12, Corning Glass Works, Corning, New York). All subsequent isolation steps were carried out at 4°C.

The blood was centrifuged at $1000 \times g$ for 20 min. The plasma and the upper 2/3 of the packed red cell layer were removed by gentle pipetting. The erythrocytes in the remaining blood were lysed by hypotonic shock with 20 ml of distilled water. Isotonicity was restored after 30 s by adding 10 ml of 2.7% NaCl in PBS. After centrifugation at $200 \times g$ for 10 min the PMN were washed twice in Eagle's balanced salt solution (Difco Labs, Detroit, USA). Cell concentration and viability were determined with a hemocytometer using 0.5% Trypan blue as the diluent. The cell suspension was then diluted with Eagle's balanced salt solution to the desired cell concentration. In the experiments designed to investigate the effect of glucose and lactose on CL, the cells were washed and suspended in appropriate media: glucose-free Hanks balanced salt solution without phenol red (HBSS) buffered with 20 mM HEPES (GIBCO, Bio AG, Basel, Switzerland) and HBSS supplemented with either glucose (1 g/l of solution), lactose (1 g/l) or both (1 g each/l).

Milk samples were taken from mammary quarters with low (<1) California Mastitis Test (CMT) scores and which were free from culturable bacteria. Polymorphonuclear leucocytes were harvested after induction of leucocytosis in a single quarter of each cow. Leucocytosis was induced after the afternoon milking by introducing 30 - 50 ml PBS containing 0.1% (w/v) oyster glycogen into the udder (13).

Twelve hours later 40 ml of milk was aseptically withdrawn into 5 ml PBS containing 1.5% EDTA in 50 ml centrifuge tube. The subsequent isolation steps were identical to those described for blood PMN except that the hypotonicity treatment used to lyse red cells was omitted.

Cell viability was >98% in both blood and milk PMN. May-Grünwald Giemsa-stained smears showed that PMN suspensions isolated from blood and milk were 90 - 95% (22 examinations) and 86 - 98% (10 examinations) pure, respectively.

Chemiluminescence measurement

Zymosan A was washed twice, suspended at a concentration of 10 mg/ml and kept frozen in small aliquots at -20°C . Luminol-BSA was prepared by stirring 4% (w/v) bovine serum albumin (Armour Pharmaceutical Co.Ltd., Eastbourne, England) with approximately 30 mg/100 ml luminol for 1 hr. The solution was then centrifuged at 25'000 xg for 20 min to remove particulate luminol. Luminol-BSA was kept frozen in small aliquots at -20°C and thawed immediately before use. The cell suspension at the desired concentration was distributed into Pico glass scintillation vials (Packard Instrument Co. Inc., Downers Grove, Ill), 0.75 ml/vial, and 15 μl of Luminol-BSA was added. Vials were

placed in a 37°C water bath in the dark for 90 minutes. CL measurements were performed in a darkened room using a Liquid Scintillation Spectrometer (Packard Tricarb Mod. 3375), operated in the "out of coincidence" mode using the settings for Tritium. To determine background counts cellular light emission in each vial was measured twice before the cells were stimulated by the addition of 50 µl zymosan suspension.

For all determinations of CL, samples were run in triplicate with one additional sample mock-stimulated with PBS. Zymosan or PBS were added to the vials at 30 second intervals, and CL was also measured at 30 second intervals; this enabled us to determine 12 samples at identical time points in 6 minute intervals. Between countings, the vials were placed in a 37°C water bath. For comparisons between blood and milk PMN, the cells were prepared at the same time and CL was measured simultaneously to avoid possible differences due to variations in incubation time before the addition of zymosan. Inhibitors, when used, were added to the cell suspensions 15 minutes before the addition of zymosan.

For the experiments designed to compare the effect of different stimuli on CL production by milk and blood PMN, the agents were prepared as follows: Sendai virus was grown in 11-day-old embryonated chicken eggs and purified as described by Peterhans (16). PMA and the Ca iono-

phore A 23187 were dissolved in dimethyl sulfoxide (DMSO) and further diluted with PBS. For stimulation we used 600 hemagglutinating units (HAU) of Sendai virus in 15 μ l PBS, 1 μ g PMA in 15 μ l PBS or 0,4 μ g A 23187 in 7,5 μ l PBS, respectively. Controls were mock-stimulated with either PBS or a corresponding amount of DMSO in PBS.

RESULTS

Effect of luminol on CL

Figure 1 shows that 6000 blood PMN were sufficient to generate a significant CL response in the presence of luminol, the CL peak reaching more than 150'000 cpm above background levels of unstimulated controls. Increasing the cell number by 3 and 9 - fold enhanced peak CL in proportion to the increment in the cell number, suggesting that the light emission by single cells was relatively constant within this concentration range. In contrast to the high sensitivity at which CL could be detected in the presence of luminol, approximately 80 times more cells (500'000 vs 6000) were needed to induce a significant CL response in the absence of luminol (increase by 5×10^4 cpm) (Figure 2).

Based on these results, it was decided to use luminol in all subsequent experiments. Preincubation of zymosan with serum (opsonization) enhanced CL by approximately 20% (not shown). We used unopsonized zymosan in all further experiments.

CL response of blood PMN compared to that of milk PMN

The CL responses of PMN isolated from blood and milk of an individual animal are shown in Figure 3. While the

kinetics of cellular light emission of blood and milk PMN were similar, the peak light emission of blood PMN was approximately 3 times that of PMN isolated from milk. When this experiment was repeated with PMN isolated from different animals, we found considerable variation in the CL ratio between blood and milk PMN. To investigate if this ratio was typical of individual animals or rather indicative of different physiological states within a given cow, PMN were isolated on repeated occasions from six animals. Figure 4 shows the results of these experiments. Counts accumulated by milk PMN over 30 minutes are given in percent of counts accumulated by blood PMN run in a parallel experiment. The variation between different animals clearly exceeds that between different cell isolates from individual animals. The ratio between milk PMN CL to blood PMN CL ranged from approx. 0.3 (in cows Nos. 1 and 3) to 1.3 (in cow No. 5).

Biochemical aspects of zymosan-induced CL in milk and blood PMN

The biochemical origin of CL has been shown to differ according to the nature of the stimulating agent as well as with the type of cell (23). Contamination by other cells was similar (not exceeding 14%) in PMN suspensions

isolated from milk and blood and can be assumed to have similar effects in experiments with either cell type. We therefore determined if various metabolic inhibitors affected CL similarly in milk and blood PMN (Figure 5). None of the inhibitors used did affect the kinetics of CL production neither in blood PMN nor in milk PMN. Results are, therefore, given in counts accumulated over 30 min. Counts in the absence of inhibitors were taken as 100%. Catalase, which dismutates H_2O_2 , did not affect CL in milk and blood PMN while superoxide dismutase decreased CL by some 45% in both cell populations. Sodium azide, which inhibits myeloperoxidase and scavenges singlet oxygen, blocked CL by >95% in both milk and blood PMN. In contrast to these agents, sodium benzoate exerted a quantitatively different effect on CL in milk and blood PMN ($P < 0.001$, Student's t test). At a concentration of 8 mM, sodium benzoate inhibited CL in blood PMN by $32 \pm 3\%$ (mean $\pm$ Standard error of the mean, SEM, $n = 7$) and in milk PMN by $60 \pm 4\%$ ($n = 5$).

To investigate the effect of glucose and lactose on zymosan induced CL, milk and blood PMN were suspended in HBSS (glucose-free) and HBSS supplemented with glucose and/or lactose. The latter was included in the experiment because it is the main sugar in milk and is

known not to be utilized by cells. CL in the presence of glucose was taken as 100%. The depletion of glucose from the medium decreased blood PMN CL significantly ($P < 0,001$) more than milk PMN CL (by $58 \pm 5,3\%$, $n = 6$ vs $13 \pm 2,8\%$, $n = 10$). The supplementation of glucose-free HBSS with lactose further decreased CL, residual light emission in both cell populations being between 35 - 40% of CL in medium containing glucose but no lactose. When glucose was added to the medium containing lactose, CL increased in both milk and blood PMN to 70 - 75% of the control with glucose alone, suggesting that lactose exerted some inhibitory effect on cellular light emission (Figure 6).

The qualitative difference in the roles of the hydroxyl radical and glucose in blood and milk PMN CL suggested that the biochemical events leading to cellular light emission might be different in these two cell populations. To obtain further evidence for such a diversity, we used different stimuli to activate the cells. PMN from milk and blood obtained on repeated occasions from cows Nos. 4,5 and 6, were suspended in HBSS containing glucose and stimulated with either zymosan, phorbol myristate acetate (PMA), Sendai virus or the Ca ionophore A 23187. While the kinetics of light emission induced by the different stimulating agents

were similar in milk and blood PMN, however, depending on the nature of the stimulus, peak light emission was reached at a different time. Thus, peak CL was reached between 2 and 6 min when the cells were stimulated with either PMA, A 23187 or Sendai virus while maximum light emission was reached between 14 and 22 min when zymosan was used as a stimulus. Relative to prestimulation levels, light emission returned to similar levels at 30 min with PMA, A 23187 and zymosan as it did at 20 min with Sendai virus. Therefore, counts accumulated over 30 min with PMA, A 23187 and zymosan and over 20 min with Sendai virus are shown in Figure 7. Counts accumulated by milk PMN are given in % of counts accumulated by blood PMN in response to the same stimulus. The ratios between blood PMN CL and milk PMN CL were clearly different. While milk PMN generally reacted to a lesser extent than did blood PMN when stimulated with zymosan, PMN and the Ca ionophore A 23187, they showed a more pronounced CL response than blood PMN when exposed to Sendai virus.

DISCUSSION

Assays of cell activity should depend on parameters which are sensitive, easy to measure and which are clearly related to a particular function of the cell. Of these criteria, the measurement of chemiluminescence (CL) meets only the first two while the functional significance of cellular light emission is not well defined. As a step in this direction, we have investigated the chemiluminescent response of bovine PMN. The approach chosen was twofold: firstly, using one stimulus, zymosan, we compared quantitative and qualitative aspects of CL generation from PMN isolated from milk and blood. Secondly, the chemiluminescent response of these cells was compared using several receptor - dependent and -independent stimuli. Cells isolated from milk and blood were chosen as examples of presumably identical cells with different bacteria killing efficiencies (8, 15,17,21).

Previous studies on bovine PMN CL required some 10^7 cells per vial to obtain a significant CL response. Using luminol as an amplifier of CL, a substantial CL response could be demonstrated with as little as 6000 cells/vial (Figure I) (1,2). In addition, CL measurement also gave reproducible results as seen from the quantitative comparison of milk and blood PMN CL (Figure 4). Similar

individual differences in milk and blood PMN function have been observed earlier from phagocytosis and bacteria killing assays (14).

To investigate the contribution of different oxygen species, we used different metabolic inhibitors, known to affect the bactericidal but not the phagocytic activities (10,25). It should be stressed that the scavengers used give informations on the contributions of different forms of "activated" oxygen to CL at locations accessible to the scavengers rather than on the absolute percentage at which the oxygen species are produced by the cells. Different from human PMN, H_2O_2 seemed not to contribute to CL in bovine PMN (2,10,25) (Figure 5). The effects of superoxide dismutase (SOD) and sodium azide suggest that O_2^- and myeloperoxidase (and possibly singlet oxygen) contributed significantly and to a similar extent to CL production in cells isolated from milk and blood. In contrast to the results obtained with catalase, SOD and sodium azide, sodium benzoate, a scavenger of $\cdot OH$, reduced CL in milk PMN significantly more than in blood PMN (60 % vs 32 %) (Figure 5). No evidence was found that the spectrum of oxygen species leading to CL was different between individual animals neither in blood nor in milk PMN.

As a further point of difference between milk and blood PMN CL, the latter was clearly more dependent on the

presence of external glucose. A possible explanation for this difference is that glucose (perhaps originating from dying cells) was present at higher concentrations in preparations of milk PMN compared to blood PMN. However, 3-O-methylglucose, used to block the utilization of "free" glucose, did not alter the different glucose requirement for CL in milk and blood PMN (not shown). It is interesting to note that the reactions of the classical respiratory burst depend on glucose while no direct requirement for glucose so far has been demonstrated for the reactions of the arachidonic acid cascade. Thus, one could speculate, that the latter reactions, leading to the production of important mediators such as prostaglandins and leucotrienes, are more prominent in milk PMN. However, proof for this interpretation is lacking and must be obtained by direct assessment for such products.

The reason for the inhibitory effect of lactose on CL generation by both cell populations is not known and remains to be determined. However, a secondary quenching effect seems unlikely, because increasing the lactose concentration in the reaction medium from 2.8 mM to 13.9 mM did not enhance the inhibitory effect on CL generation (not shown).

In the experiments discussed so far, the same agent, zymosan, had been used to activate the cells. As it has

been shown that CL depends on the cell type as well as on the nature of the stimulating agent (23), it was of interest to compare the chemiluminescent response to different agents. Phorbol myristate acetate (PMA) and the calcium ionophore A 23187 activate cells by receptor-independent mechanism(s) while Sendai virus, although not a bovine pathogen, can bind to and replicate in bovine cells (22). As shown in Figure 7, the CL response to these agents by milk PMN was different from that by blood PMN, further indicating that different biochemical events, at least in a quantitative manner, lead to CL in these two cell populations.

Taken together, the results demonstrate a surprising divergence in the CL response of bovine milk and blood PMN to zymosan as well as to other agents able to activate PMN. This divergence comprises quantitative as well as qualitative aspects of cellular light emission. Clearly, the experiments reported here do not explain these differences. However, they show that CL measurement is a useful tool to detect differences between cell types and they also suggest a direction for future research. Obviously, interpretations as to "good" or "poor" PMN function based on CL measurement must be made with caution. To change CL from an ill defined to a useful parameter of PMN function it is necessary to extend the definition of reactions contributing to CL beyond basic properties such as glucose

dependency. Furthermore, it is essential to investigate if the activation of particular reactions leading to CL can be attributed to a given stimulant.

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Figure 1

The effect of cell concentration on CL in the
presence of luminol

PMN were isolated from blood as described in materials and methods and suspended at various cell concentrations in Pico glass scintillation vials (volume 0.75 ml). The assay medium contained 15 μ l luminol/vial. After adaption to the dark at 37°C for 90 minutes, 0,5 mg (50 μ l) zymosan was added to each vial and CL was measured for 0.2 min periods at 6 min intervals. Between countings, cells were incubated in a water bath (37°C). Zero counts represent background CL measured before the addition of zymosan. Samples were run in triplicate, bars represent standard deviation.

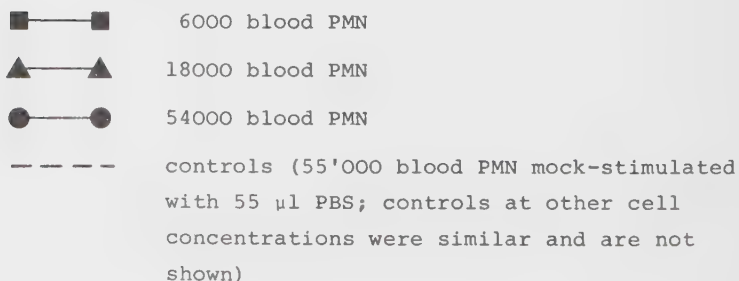


Figure I

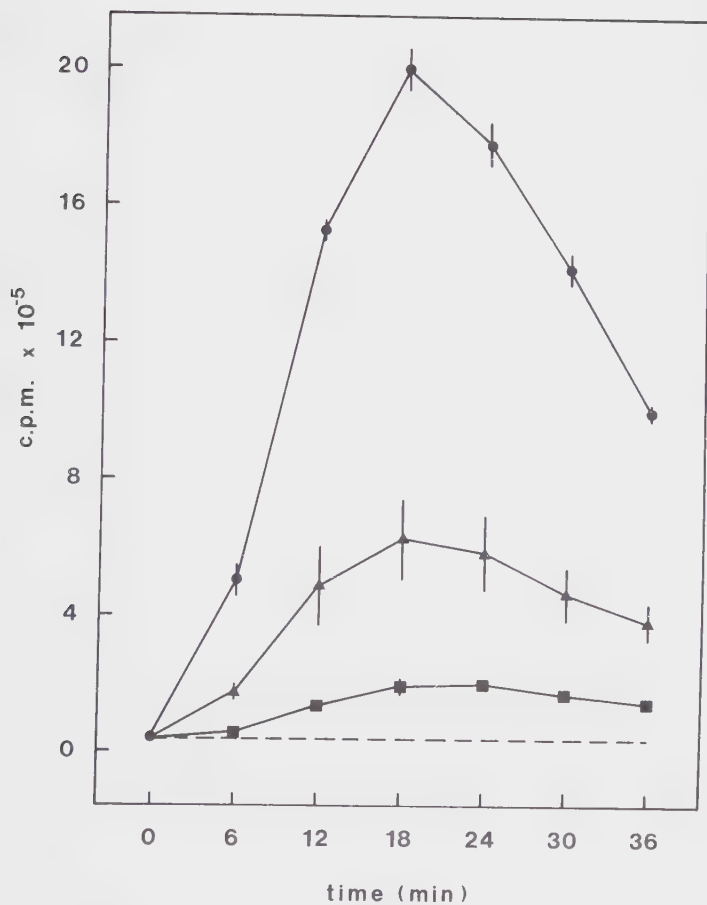


Figure 2

The effect of cell concentration of CL in the absence
of luminol

The experiment was performed as described in the legend
to Fig. 1 except that luminol was absent from the assay
medium.

■ — ■	0.5×10^6 blood PMN
▲ — ▲	2.0×10^6 blood PMN
● — ●	5.0×10^6 blood PMN
---●---	control (5×10^6 blood PMN)

Figure 2

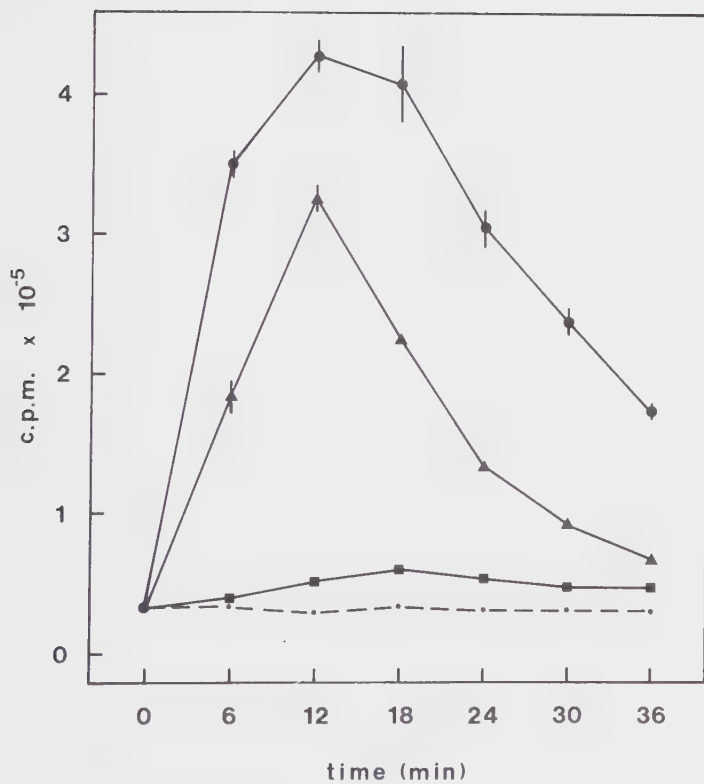


Figure 3

Comparison of the Luminol-dependent CL response of
blood and milk PMN

Blood and milk PMN were isolated from cow No. 3, suspended at a concentration of 500'000 cells/Vial and stimulated with 0.5 mg zymosan. CL was measured as described in the legend to Fig. 1.

Each point represents the mean of 3 samples run in parallel; bars represent standard deviation.

△ — △ milk PMN
○ — ○ blood PMN
--●--● controls (blood PMN mock-stimulated with PBS)

Figure 3

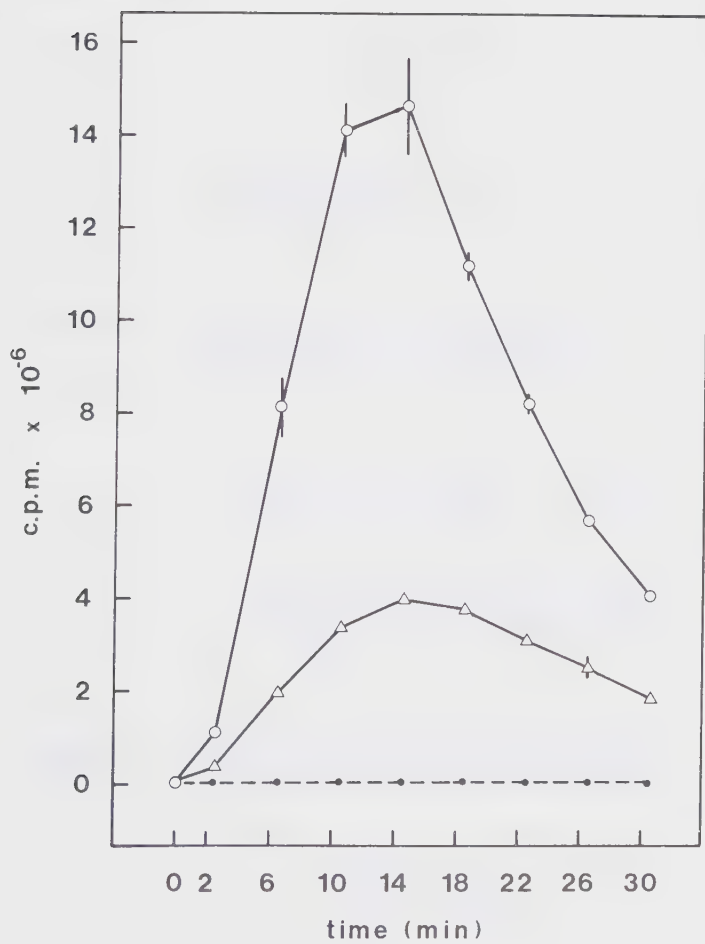


Figure 4

Comparison of milk PMN and blood PMN CL from six
different cows

PMN were isolated simultaneously from milk and blood, suspended at a concentration of 500'000 cells/vial and stimulated with 0,5 mg zymosan. CL was measured as described in the legend to Fig. 1. The total counts accumulated in the 30 minute period following stimulation were calculated from the areas under the light emission curves. Milk PMN CL is expressed in % of corresponding blood PMN CL. Bars represent standard error of the mean (SEM).

Figure 4

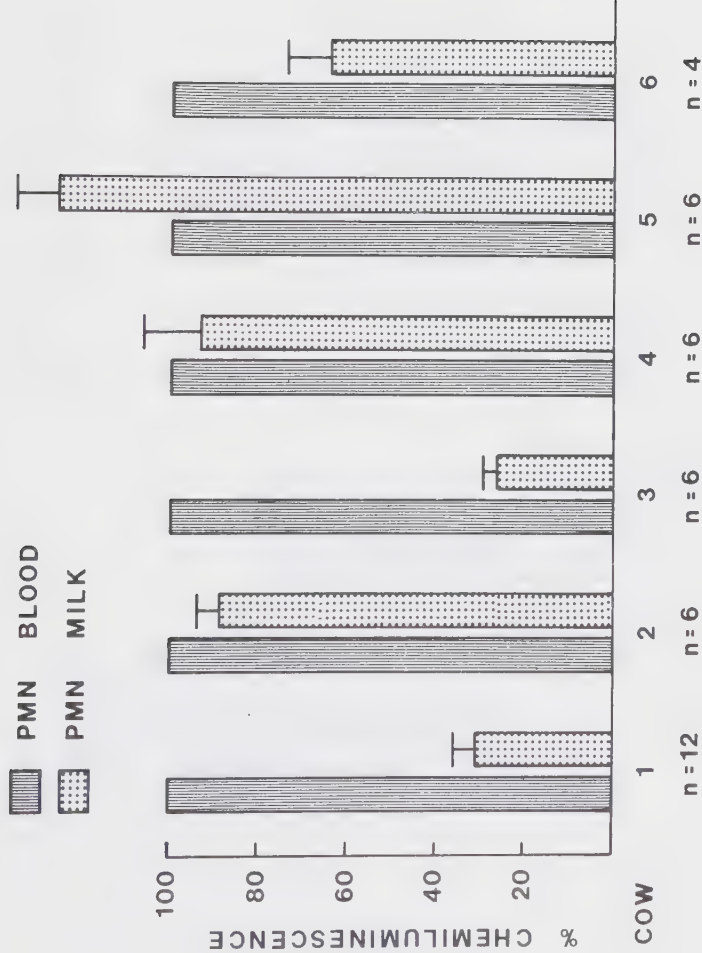


Figure 5

The effect of different inhibitors in the CL response
of blood and milk PMN

PMN were isolated from 12 different cows, stimulated with zymosan and CL was measured as described in the legend to Fig. 1. Inhibitors were added 15 min before zymosan. Columns represent counts accumulated over a period of 30 min following stimulation (determined as in Fig. 4) and are expressed in % CL of untreated controls. Bars represent SEM.

Figure 5

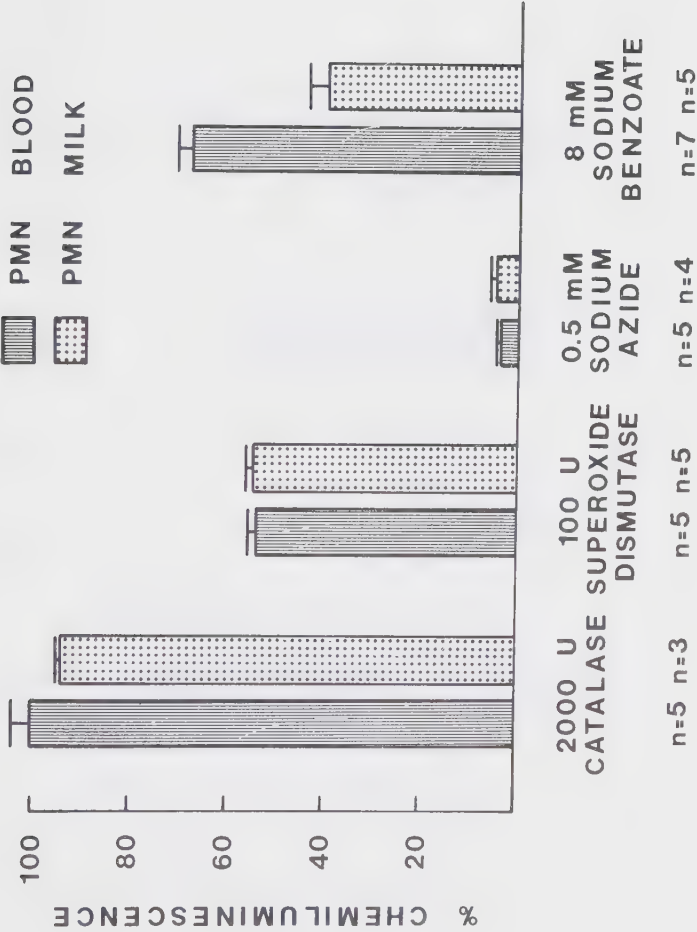


Figure 6

The effect of glucose and lactose on CL by milk and
blood PMN

PMN were isolated from 16 different cows and suspended in appropriate media as described in materials and methods. The kinetics of CL production were similar in milk and blood PMN and in all media used. Columns represent counts accumulated over a period of 30 min following the addition of zymosan and are expressed in % CL of the reaction in HBSS containing glucose.

Figure 6

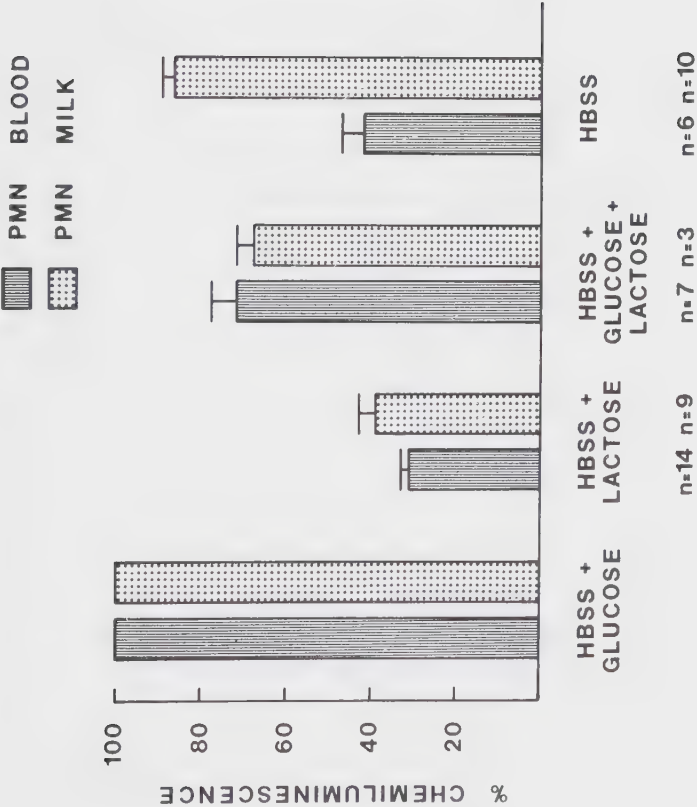
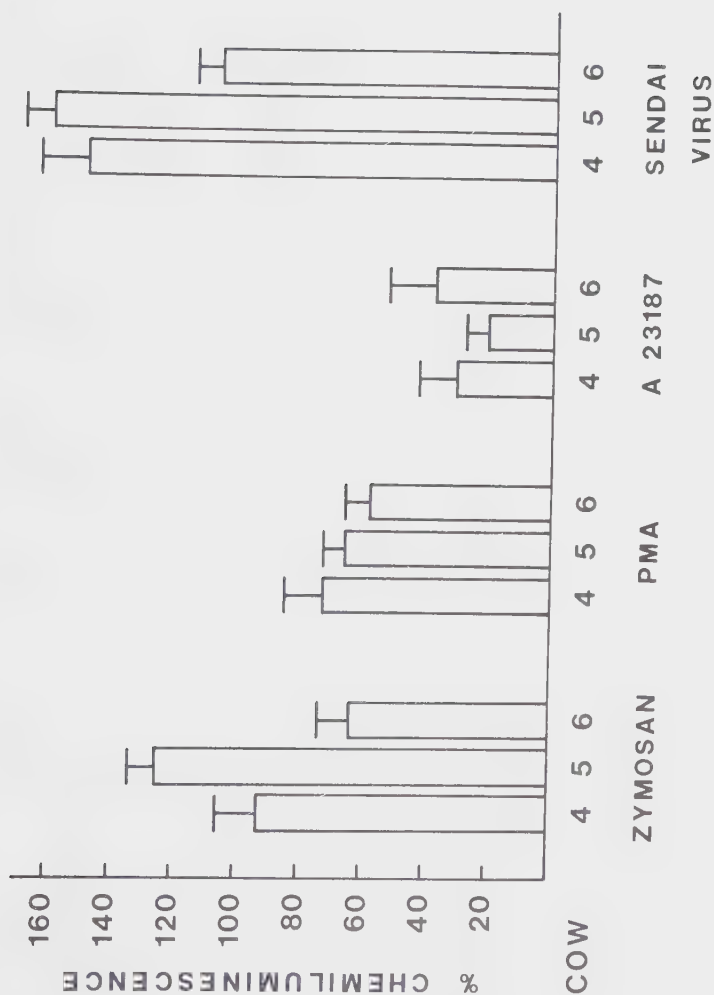


Figure 7

The effect of different stimuli on the generation of
CL by milk and blood PMN

PMN were isolated from milk and blood of cows Nos. 4, 5 and 6, suspended in HBSS containing glucose (1 g/l) at a concentration of 500'000 cells/vial and stimulated with either 0,5 mg zymosan, 1 μ g PMA, 600 HAU Sendai virus or 0,4 μ g A 23187. Counts were accumulated over a period of 30 min when stimulated with zymosan, PMA and A 23187, and 20 minutes when stimulated with Sendai virus (see results section). Columns represent milk PMN CL in % of corresponding blood PMN CL. Bars represent SEM (n = 6 in cows Nos. 4 and 5, n = 4 in cow No. 6).

Figure 7



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